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Disposition and pharmacokinetics of azithromycin in serum and a lung tissue of two modified-release formulations compared with an immediate-release product on the market

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Abstract: The aim of this study was to determine the disposition and pharmacokinetics in serum and a lung tissue homogenate in guinea pig (*Cavia porcellus*) of two experimental formulations of azithromycin, those were included in a modified release polymer matrix (MRF) after oral administration. The results obtained are compared with a commercial form of immediate release. 3 groups of animals were randomly formed in groups of 7 for control and 14 for each group of modified-release formulations (MRFs) were treated with a single dose of 8mg/kg of active principle. In lung tissue, comparisons of concentration of azithromycin, showed statistically significant differences between commercial product, MRF1 and MRF2. All pharmacokinetic parameters for MRF1 and MRF2 were significantly different with the exception of C_{max} with respect to commercial product. The treatment of the animals with MRFs may have several benefits over treatment with azithromycin alone since could increase dosing interval for the two MRFs evaluated and reduce the frequency of application, patient stress levels and toxicological risks by accumulation of the active principle.

Keywords: Azithromycin, flip flop pharmacokinetic, macrolide, modified release.

INTRODUCTION

Azithromycin (AZT) is an antimicrobial in the macrolide family. It is rapidly absorbed orally, reaches the maximum plasma concentrations in a very short period and is widely distributed in the body, except in the brain and cerebrospinal fluid (Bakheit *et al.*, 2014; Parnham, *et al.,* 2014). AZT has a wide cellular distribution (including phagocytes), resulting in much higher drug concentrations in tissues or secretions compared to the plasma and achieving an extended tissue half-life (Davis *et al.*, 2002). Azithromycin is metabolized in the liver by demethylation to produce inactive metabolites. Bile is the main route of elimination, with 50% of the drug excreted unchanged, and 12% of the drug is excreted unchanged in the urine (Viluksela *et al.*, 1996). Other qualities of AZT include its resistance to the acidic pH of the stomach, rapid oral bioavailability, partial metabolism, slow elimination and residual behaviour, all of which increase its half-life. (Bakheit *et al.*, 2014) Azithromycin has activity against Gram-positive and Gram-negative microorganisms, such as *Mycoplasma pneumoniae, Borrelia burgdorferi* and *Toxoplasma* spp. (Parnham *et al.,* 2014) and it reportedly even has effectiveness against *Giardia intestinalis* infections. (Zygner *et al.,* 2008) AZT has a predominantly time-dependent effect with a post-antibiotic effect of 8 hours or more. Its mechanism of action is through the inhibition of protein synthesis by reversibly binding to the V domain of 23S ribosomal RNA (Plumb, 2015; USP, 2014). According to the pharmacokinetic/pharmacodynamic (PK/PD) relationship of Azithromycin and its time-dependent action, the present work suggests the possibility of designing an oral preparation with an initial fraction that is immediately released and a second prolonged release phase to achieve therapeutic levels rapidly and maintain them for an extended time, which could improve the antibacterial effects and reduce the side effects (Sinko and Singh, 2011). The goal of the present study is to carry out the preclinical tests of the new formulations designed for end use in dogs, determine the disposition and concentrations of AZT in guinea pig serum and a lung tissue homogenate after the oral administration of a modified-release tablet compared to a commercial immediate-release form.

MATERIALS AND METHODS

Azithromycin dihydrate (CAS number 117772-70-0) with a molecular weight of 749 and a purity of 98.5µg/mg acquired from Antvol Chemicals, México; Acrylic acid polymer (Carbopol® 971 P NF polymer) Lubrizol, Mexico; Alginate of sodium; Maltose; Magnesium stearate and Hydroxy propyl methyl cellulose (Methocel® K100) Methocel, Mexico were used.

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Pre-formulation stage

The rheological properties of the powder mixture were evaluated, included apparent density, compressed density, true density, Carr compressibility index, Hausner ratio, porosity percentage, angle of repose and flow velocity. The wet percentage of the powder was measured with a thermobalance. MB 2000, Ohaus Corp. Parsippany, NJ.

All techniques were performed according to the US Pharmacopeia (2014). From each formulation, the tablets were prepared and diameter, thickness, brittleness and hardness were determined.

Modified-release formulations (MRF)

The two formulations proposed were prepared in a clean and controlled area in the laboratory of pharmaceutical technology of the Faculty of Chemistry of the National Autonomous University of Mexico and designed in tablet form by direct compression in a hydraulic press Carver 4350, USA. The preparations were preserved separately in new amber bottles in a temperate and dry climate. The present trial used in the two formulations Methocel as the main binder of the tablets and the main source of the formation of the matrices, in the first formulation carbomer was incorporated as a binder and a modifier of the mucoadhesive characteristics in the second formulated Sodium Alginate was used as a tablet viscosifier to facilitate the formation of the hydrophilic matrix by retarding the release of the active ingredient (Sinko and Singh, 2011). These formulations were made with the purpose of modifying and extending the release of the active ingredient. In both cases, the formulation for the development of a tablet was adapted by including a diluent (Direct Compression Maltose) and a lubricant (Magnesium Esterate) according to the parameters described by the literature (Rowe *et al.,* 2009; Sakr and Alanazi, 2012). AZT powders were subjected to compression under 10000lb of pressure, forming a tablet of 1.3mm in diameter, 3mm wide and with 500mg of Azithromycin on average.

The modified-release formulation 1 (MRF1) was designed with the ingredients: AZT (25%), HPMC K100 M, Sodium alginate, Magnesium stearate, and Maltose in the following proportions: 1:1.8:0.08:1.11:0.01. Modified-release formulation 2 (MRF2) was designed with AZT (25%), HPMC K100 M, Carbopol 71G, Magnesium stearate, and Maltose in the following proportions: 1:1.8:0, 173: 1.016:0.01. In both cases, the ingredients were incorporated according to the parameters established in the literature (Rowe *et al.,* 2009).

Animals (care and dosage)

This study was approved by the Institutional Commission for Research, Attention and Use of Experimental Animals (SICUAE) of the National Autonomous University of Mexico, according to Official Mexican Regulation NOM-062-ZOO-1999.

Thirty-five guinea pigs of different sexes that were clinically healthy and obtained from the Department of Chemical and Biological Inspection of the Facultad de Medicina Veterinariay Zootecnia of the National Autonomous University of Mexico were used. The animals weighed approximately 521g and were 3 months of age on average. They were fed Guinea Pig® Purina, Mexico, had free access to drinking water, and had not received medication for at least 30 days before the test. Three groups of guinea pigs were randomly formed. The administration dose was 8mg/kg per the active principle.

Group 1 (n=7) received an oral tablet of a commercially available Azithromycin (CP). Group 2 (n=14) received a tablet formulated with MRF1, and Group 3 (n=14) received a tablet formulated based on MRF2.

Collection and analysis of the samples

Blood samples were collected from one guinea pig per time in each group by direct cardiac puncture under anaesthesia with sodium pentobarbital. After the collection of blood, an overdose of the barbiturate was administered to induce euthanasia. Then, 1g samples of lung tissue were obtained.

The blood samples were centrifuged at 1,500rpm for 15 minutes, and the serum was removed by placing it in Eppendorf tubes. The extraction and maceration of lung tissues resulted in samples that were cooled to -20°C until analysis.

The serum and tissue concentrations of Azithromycin were determined by a modified agar diffusion analysis using *Staphylococcus aureus* ATCC 11778. American Type Culture Collection, Manassas, Va. (Bennett *et al.,* 1966; Nunes and Ferreira, 2005) as the test organism cultured on Mueller Hinton agar. BIOXON, Becton Dickinson, Mexico.

To determine the toxicological effects of the administration of the different formulations, blood samples were sent to the Department of Pathology of the Facultad de Medicina Veterinaria y Zootecnia of the National Autonomous University of Mexico, where haematological and blood chemistry studies were carried out at different times.

Pharmacokinetic analysis

A computerized curve-stripping program was used PK Analyst, Micromath Scientific Software, Salt Lake City, UT, USA to analyze AZT concentration versus time curve for each individual animal after the oral administration of the MRF1, MRF2, and CP. The Akaike information criterion and graphical analysis of weighted residuals were used to determine the optimal pharmacokinetic model. (Wagner, 1993) For oral administration, fitted curves of Azithromycin dihydrate showed the decreased

pulmonary tissues drug concentrations as a function of time and were approximated to one compartment with first-order input and first-order output with the following equation ($R^2 > 0.9$):

$$C(t) = \left[\frac{(Dose \times K_{ab})}{(Volume K_{ab} - K_{el})} \right] \times (e - K^{el \times time}) - (e - ^{Kab \times time})$$

Where C (t) is the concentration as a function of time, e is the base of the natural logarithm, K_{ab} is the absorption rate constant, K_{el} is the elimination rate constant, and t is the time since the drug was administered.

The following pharmacokinetic parameters were obtained with a computerized curve-stripping program: elimination half-life (t½), the maximum plasma concentration (C_{max}), the area under the curve (AUC), the area under the concentration-time curve calculated by the trapezoidal method (AUC_t), the area under the first moment of the concentration-time curve (AUMC) and the retention time (RT). The maximum plasma concentration time (T_{max}) was determined by inspecting the concentration in the macerated lung tissue and determining the concentration-time profiles of Azithromycin. The mathematical determination of the apparent volume of distribution at steady state was conducted using the following equation: (Wagner, 1993)

$$Vd_{ss} = \frac{(Dose \times AUMC)}{AUC^2}$$

The half-life was obtained using the following formula:

$T ½ = 0.693 / K_{el}$

The total clearance (Cl_b) of oral Azithromycin was determined by

Cl_b=Dose / AUC

The AUC∞ was calculated as follows:

$AUC_{0-\infty} = AUC + (C_{last} / K_{el})$

The relative bioavailability (Fr_{el}) was calculated with the equation

Fr_{el} = (AUC ERF / AUC PC) x100

Fr_{el} was expressed as the percentage of the AUC of the experimental group relative to the control group.

STATISTICAL ANALYSIS

The number of animals used for the study (n=35) was determined using the method described by Montgomery (2004) for an experimental design of one factor (Concentration of azithromycin in lung and serum, after oral administration of the Active principle included in two different hydrophilic matrix formulations in tablet form and their contrast with a commercial product), The normality and the uniformity of the data was determined by the Shapiro-Wilk test. The study focused on the determining the minimum significant difference at a significance level of α = 0.05 by analysis of variance of a factor and subsequent contrast using the Dunnett T test with a focus on the differences in C_{max}, final concentration, AUC and The magnitude of the concentration differences at time 6 hours and 24 hours. using the statistical software of Excel from Windows 10.0.

RESULTS

The rheological properties of the powder mixture were: The Apparent density of the MRF1 mixture was 0.587 g/cm^3, the apparent density was 0.530±0.001g/cm^3 and the true density was 0.682g/mL. The Carr index was 23.4 ±0.6, Hausner's radius was 1.30±0.06, the porosity percentage was 1.3±0.6, the angle of repose was 17.63± 0.17° and the moisture content of the powder was 3.31% ±0.25. For MRF2, the density was 0.549g/cm³, the

Table 1: Pharmacokinetics values of lung tissue homogenate derived from 35 healthy adult guinea pigs randomized to receive a single oral dose of azithromycin dihydrate in a commercial presentation (control treatment) or of the experimental modified-release formulations (MRF1, MRF2). Within each row, values without a common superscript letter differ significantly with a value of ($P < 0.05$)

Parameter	Control treatment	MRF 1	MRF2
$K1/2_{elim}$ (h)	19.9 ± 3.46	91.2 ± 11.95	44.6 ± 1.3
$K1/2_{ab}$ (h)	1.12 ± 0.19	0.35 ± 0.04	0.35± 0.01 [b]
T_{max} (h)	4.93 ± 0.86	2.85 ± 0.37	2.49 ± 0.07 [b]
C_{max} (µg / mL)	20.9 ± 3.7	21.93 ± 2.9 [a]	16.44 ± 0.5 [b]
AUC (µg*h/mL)	668.5 ± 116.7	2118.8 ± 277	128.3 ± 37.8
Residence Time (h)	302.6 ± 52.8	1321 ± 173.1	648.9 ± 19.2
Trapezoidal AUC (µg*h/mL)	694.0 ± 121.2	1910 ± 250.3	1291.3 ± 38
Vd_{ss} (L)	36224 ± 6323	49886 ± 6536	4058.5 ± 119
Cl_b (L/h)	0.0006 ± 0.0001	0.0000013 ± 1.7E-07	0.0001 ± 2.95E-06
AUC∞ (µg*h/mL)	6723 ± 1173	11846.5 ± 1552.3	978.3 ± 28.9
Fr_{el} (%)	-	3169.5 ± 415	1913.6 ± 56.5
K_{el} (µg/mL^{-1})	0.6188 ± 0.1	19.8 ± 2.6	19.8 ± 0.58 [b]
K_a (µg)	16.322 ± 2.8	0.27 ± 0.0003	0.186± 0.005
K_a/K_{el}	0.3791 ± 0.06	73.3 ± 966.2	106.7 ± 3.2

apparent density was 0.526±0.006g/cm³ and the true density was 0.682g/ml. The Carr index was 23.4±0.87, Hausner's radius was 1.31±0.06, the porosity percentage was 1.31±0.06, the rest angle was 9.62±0.17° and the moisture content of the powder was 3.31%±0.25.

The tablets of MRF1 had an average weight of 0.5015g ± 0.001, a diameter of 12.98mm±0.008, and a thickness of 3.01mm±0.0032. The fragility was 1.0006% ± 0.005, and the hardness was 13.4666 Kp. The MRF2 tablet had an average weight of 0.5014g±0.001, a diameter of 12.97 mm ± 0.0023, a thickness of 3.07mm±0.004, a fragility of 0.5996% ± 0.003 and a hardness of 18.6666 Kp.

The concentrations in serum and lung tissues are showed in fig. 1. The pharmacokinetic values of each modified-release formulations and of the control treatment are summarized in table 1 and 2. At the end of the study no adverse effects or mortality were detected in the animals. Based on the results of hematological and blood chemistry evaluations did not show acute toxicity after treatments summarizes in tables 3 and 4.

DISCUSSION

The purpose of the design of these MRFs of Azithromycin dehydrate, was to offer with a single dose administration, an adequate therapeutic value for a maximum time, thus providing the opportunity to maintain the minimum inhibitory concentrations (MICs) of AZT. Whose antimicrobial efficacy depends on maintaining the indicated concentration range over time. Adequate formulation in clinical practice will help reduce the incidence of adverse side effects derived from peak

Table 2: Serum Pharmacokinetics values derived from 35 healthy adult guinea pigs randomized to receive a single oral dose of azithromycin dihydrate in a commercial presentation (control treatment) or of the experimental modified-release formulations (MRF1, MRF2). Within each row, values without a common superscript letter differ significantly with a value of ($P < 0.05$).

Parameter	Control treatment	MRF 1	MRF2
$K1/2_{elim}$ (h)	3.9 ± 0.27 [a]	13.183 ± 3.4 [b]	12.439 ± 4.1 [b]
$K1/2_{ab}$ (h)	1.186 ± 0.18 [a]	2.808 ± 0.11 [b]	1.2388 ± 0.11 [a]
T_{max} (h)	1.1528 ± 0.95 [a]	7.63 ± 0.31 [b]	4.358 ± 0.17 [a]
C_{max} (µg / mL)	1.1211 ± 0.5 [a]	0.918 ± 0.12 [a]	1.0469 ± 0.19 [a]
AUC (µg*h/mL)	6.3084 ± 0.9 [a]	18.47 ± 1.1 [b]	17.788 ± 3.3 [b]
Residence time (h)	5.62 ± 0.77[a]	19.02 ± 3.3 [b]	17.946 ± 4.2 [b]
AUC Trapezoidal (µg*h/mL)	12.32 ± 0.51 [a]	23.78 ± 4.3 [b]	20.22 ± 5.3 [b]
AUMC(µg*h/mL)	35.49 ± 4.5 [a]	337.32 ± 20.8 [b]	332.18 ± 44.3 [b]

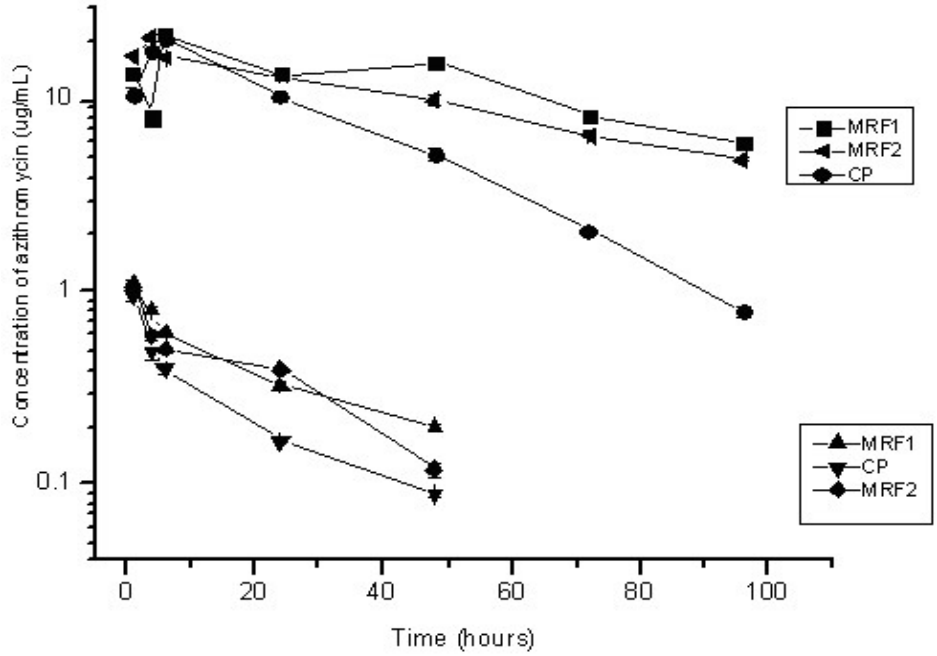

Fig. 1: Mean ± SD concentrations of azithromycin in serum and the lung tissue homogenate after the oral administration of a single dose of 8 mg/kg of azithromycin of an experimental modified-release formulations (MRF1, MRF2) and after of the administration of a commercial product (CP) available in the market.

Table 3: Mean values and standard desviation of hematology of 35 healthy adult guinea pigs administered with a single oral dose of azithromycin dihydrate in a commercial presentation (control treatment) or experimental modified release formulations (MRF1, MRF2), in times 0, 1 and 30 hours after administration

	Parameter	0	1 h	30 h	DS
Erythrocytes (10^6/µl)	4-7	6	5	5	±0.577
Hemoglobin (g/dl)	11-17	15	13	16	±1.52
Hematocrit (%)	33-45	40	38	38	±1.15
Leukocyte (10^3/µl)	7-14	9.65	10.42	9.13	±0.62
Neutrofphil (mm^3)	2000-6000	5656	5267	4579	±545.37
Limphocyte (mm^3)	3000-8000	2764	3679	2789	±521.21
Monocyte (mm^3)	200-2000	1235	1476	1767	±266.39

Table 4: Mean values and standard desviation of blood chemistry of 35 healthy adult guinea pigs administered with a single oral dose of azithromycin dihydrate in a commercial presentation (control treatment) or experimental modified release formulations (MRF1, MRF2), in times 0, 1 and 30 hours after administration

	Parameter	0	1 h	30 h	DS
Glucose (mg/dl)	50.82-216.89	110.12	205.76	226.23	±51.20
Albumin (g/dl)	0.12-2.50	0.86	0.57	1.12	±0.27
Globulin (g/dl)	0.03-1.79	0.78	0.57	1.12	±0.27
ALP (UI/l)	40.31-126	70	112	56	±29.14
GGT (UI/l)	5.94-20.06	12.34	7.56	15.67	±4.076
AST (UI/l)	18.01-106.02	75.15	87.12	57.86	±14.71
ALT (UI/l)	25.04-100.01	57.65	75.68	67.35	±9.02
Urea (mg/dl)	27.04-101.72	35.46	87.98	56.76	±26.41
Uric acid (mg/dl)	0.24-4.37	2.34	3.12	2.35	±0.44

plasma concentrations, reduce the workload of clinicians and homeowners, reduce costs and reduce stress in patients. In the present study, in lung tissue, comparisons of concentration in the last reading showed statistically significant differences between commercial product (CP) and MRFs, although the presence of AZT was found up to 96 hours in all cases, was for CP of 0.82µg/mL, while for the MRFs the concentration was exceeded 6 to 7 times more, so MRF1 was: 5.53 and MRF 2 was: 4.906µg/mL (table 1).

With respect to the absorption and removal constants (Ka/Kel) and their relationship, the results allow an inference of the kinetic behavior of flip-flops for both proposed formulations (MRF1 y MRF2), since they showed the following values: MRF1: 73.3 >2 and MRF2 106.7 >2, in both cases the rate of absorption is greater than the rate of elimination, with a value greater than 2. (Jambhekar and Breen, 2009) Unlike CP: 0.37 <2, with a value less than 2, therefore, by fulfilling a flip-flop condition, these agents can be considered candidates for use in long-lasting formulations because the rates of absorption in the long-acting formulations are greater than the rates of elimination (Yanez *et al*., 2011). The two proposed presentations showed much lower elimination rates than absorption rates, explaining the possible presence of flip-flop kinetics and unusual relative bioavailability values (Brayden 2003; Abdul *et al.,* 2010).

Clearance (Cl) is a parameter indicating the ability of an organism to remove an active substance as a function of time, expressed as the amount of plasma from which the drug has been removed per unit of time. In the present study, the values obtained were very low, indicating a slow elimination (Jambhekar and Breen, 2009). In contrast, the active substance was rapidly absorbed, reaching high concentrations in lung tissue showing a high tissue selectivity that contributes to high concentrations of Azithromycin in the respiratory tract, Gynecological tissue and prostate, remaining above the MIC for several days with the potential release of the antibiotic at sites of local infection (Hoepelman and Schneider, 1995). Although this phenomenon was observed for the three compounds, the concentration analysis over time demonstrates the effect of the hydrophilic matrix on the property of extending the release time of the active principle, maintaining ranges higher than the immediate release formulation,

demonstrating. The permanence of the formulation in the anterior part of the gastrointestinal tract (stomach or duodenum) with the consequent increase of the systemic bioavailability of the active ingredient included in a hydrophilic matrix (Carvalho *et al.,* 2010). A second characteristic is to promote the retention of the product by delaying its expulsion through different mechanisms such as bioadhesion, flotation, swelling or a combination of any of these (Brayden, 2003). Bioadhesion is the ability of adhesion to the surfaces of the mucous membranes (cell layer) or to the mucin layer coating this surface membrane. These phenomena improve residence time, which is often a problem caused by stomach emptying, peristalsis and displacement by ciliary movement at dosing (Abdul *et al.,* 2010). In the present study, combinations of hydroxypropylmethylcellulose-sodium alginate and hydroxypropylmethylcellulose-carbopol, both compounds with excellent bioadhesive characteristics (Brayden, 2003, Carvalho *et al.,* 2010) were used to obtain the mucoadhesive property and to increase the viscosity of the gel formed in that of the formulation for Control the release of the active ingredient and increase the residence time in the digestive tract.

This allows prolonged release, avoiding the presence of plasma concentration peaks and ensuring the extension of the Azithromycin uptake period to improve drug dosage (Brayden *et al.,* 2010). The relationship between release control and increased residence time in the anterior portion of the gastrointestinal tract demonstrated increased bioavailability of Azithromycin, showing better use of the administered dose compared to the same dose of the antibiotic included in three different types of excipients.

Two types were composed of hydrophilic matrices and had very similar C_{max} values, but marked differences in concentrations at the end of the trial, maintaining serum drug concentrations consistent with optimal therapeutic levels over an extended period of time (Brayden, 2003). This demonstrates the feasibility of the two proposed formulations with respect to the form of immediate release available on the market.

The literature reports that in vitro tests have shown that Azithromycin at concentrations of 2.0μg/mL or higher is effective in 90% of gram-positive aerobic microorganisms (Streptococci (Groups C, F, G), Viridans streptococci group), gram-negative aerobes, anaerobic microorganisms and others such as *Treponema pallidum and Ureaplasma urealyticum*. (Giguere, 2013) Considering the MICs of Azithromycin dihydrate and its time-dependent antimicrobial characteristics, the best pharmacokinetic profile would be achieved when the tissue concentrations of the drug are never lower than the MIC recommended for the infecting microorganism during the dosing interval (Craig, 1998). Based on this criterion, the commercial product used in this test should be administered every 24 hours, but this poses risks of accumulation and the presence of toxicity, as reported in the literature (Atli *et al.,* 2015). The dosing interval for the two modified-release preparations evaluated in the present trial could increase its range of administration and reduce the frequency of application, patient stress levels and toxicological risks by accumulation of the active principle.

The technique of quantitative / qualitative microbiological diffusion used in the present study allowed us to determine the serum concentrations of Azithromycin with sufficient reliability. This is one of the main methods of macrolide determination (Nunes and Ferreira, 2005, Bekele and Gebeyehu, 2012) and allows the determination of the active fractions of the antimicrobial agent, the inference of conclusions on the relations between the different concentrations in the serum, their efficacy and dosage intervals for specific pathogens. Therefore, it was possible to demonstrate that the proposed formulations guarantee the MICs in the time necessary for the successful antimicrobial treatment of Azithromycin-sensitive pathogens in the present study.

CONCLUSION

Extensive research has been conducted on the dosage forms of prolonged-release AZT for oral use and local application in humans, but no presentations with these characteristics have been designed in the veterinary field. The determination of the concentrations of AZT in the present study in the lung tissue of guinea pigs for 96 hours was conducted to obtain more information on the viability of use, extending the permanence of the active principle in the body and reducing the number of doses required in different treatments with this antibiotic. Undoubtedly, clinical trials and toxicological studies are needed to assess whether these preparations can be considered potentially useful in the target species. In terms of the proportions of used excipients and the active principle, they do not reach the toxic doses reported in the literature.

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